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Ammonium Metabolism in the Green Hydra Symbiosis

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Abstract. Inhibitors of enzymes of ammonium assimilation were used to test if assimilation of ammonium in the green hydra–*Chlorella* symbiosis was due to host or symbionts. Both methionine sulphoximine (MSX, an inhibitor of glutamine synthetase, found in both host and symbionts) and azaserine (AZS, an inhibitor of 2-oxoglutarate amido transferase, not found in the host) inhibited ammonium uptake by the intact symbiosis. MSX was taken up and caused predictable changes in pools of glutamate and glutamine in both freshly isolated symbionts and cultured ex-symbiotic *Chlorella*. However, after treatment of the intact symbiosis with MSX, no MSX was found in the symbiotic *Chlorella*, and glutamine and glutamate pools of both host and symbionts were unaffected. Although both MSX and AZS inhibited ammonium uptake by *Chlorella*, MSX caused seven times as much ammonium release from the intact symbiosis as did AZS. AZS treatment of the intact symbiosis caused an increase in glutamine pools in both host and symbionts, and AZS also competitively inhibited glutamine uptake by *Chlorella*. Further, ammonium treatment of intact hydra did not affect the nitrogen status of the algal symbionts, although it did cause a small increase in the number of algae in each digestive cell of the host. It is suggested that primary ammonium assimilation in the green hydra symbiosis occurs by means of animal glutamine synthetase, and that the resulting glutamine may be taken up and further processed by the symbiotic algae. Freshly isolated symbionts were able to process glutamine into glutamate even when incubated at low pH, which causes them to release a substantial proportion of fixed carbon as maltose.

Introduction

Invertebrate-microalgal symbioses are able not only to reassimilate catabolically produced ammonium but also to take up and assimilate ammonium from the environment (Kawaguti, 1953; Cates and McLaughlin, 1976; Szmant-Froelich and Pilson, 1977; Muscatine and D'Elia, 1978; Muscatine *et al.*, 1979; Wilkerson and Muscatine, 1984; Wilkerson and Trench, 1986; Rees, 1986; McAuley, 1990). However, there is some controversy over whether ammonium assimilation is due to the symbiotic algae, or to the animal host, or to the combined activities of both (Miller and Yellowlees, 1989). The possibility that animal rather than algal enzymes may be responsible, in part or in whole, for assimilation of ammonium has important implications for the mechanisms by which animal hosts regulate cell division in populations of symbiotic algae, since a number of workers have suggested that the growth of populations of symbiotic algae may be nitrogen-limited (Rees, 1986; Cook and D'Elia, 1987; McAuley, 1987a).

Miller and Yellowlees (1989) pointed out that in corals symbiotic with dinoflagellates (zooxanthellae), levels of the ammonium assimilatory enzyme NADPH-glutamate dehydrogenase (NADPH-GDH) are higher in host tissue than in symbionts, although the catabolic production of ammonium probably exceeds the capacity of host NADPH-GDH (Rahav *et al.*, 1989; Falkowski *et al.*, 1993; Spencer-Davies, 1992), which has a low specificity for ammonium (Catmull *et al.*, 1987). More recently, glutamine synthetase (GS) activity was reported in giant clam (*Tridacna gigas*) and coral (*Pocillopora damicornis*) host tissues (Fitt *et al.*, 1993; Yellowlees *et al.*, 1994). Because there are relatively high levels of ammonium-assimilating enzyme in host tissues, it is possible that much of the ammonium present in natural seawater concentrations is assimilated before it reaches the symbiotic zooxanthellae (Yellowlees *et al.*, 1994).

However, several lines of evidence suggest that some if not all ammonium assimilation is due to the zooxanthellae. Assimilation is light dependent and does not occur in aposymbiotic animals (for review, see Rees, 1986).

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Rates of uptake by intact associations are similar to those of freshly isolated symbionts (D'Elia *et al.*, 1983; Wilkerson and Trench, 1986), and intact associations exhibit uptake kinetics which suggest that symbionts assimilate ammonium from seawater by depleting ammonium in host tissue and causing a diffusion gradient to form (D'Elia and Cook, 1988). Finally, addition of ammonium to seawater causes increases in zooxanthellar mitosis, biomass, and amino acid levels (Cook *et al.*, 1988; Muscatine *et al.*, 1989; Hoegh-Guldberg and Smith, 1989; Dubinsky *et al.*, 1990; Fitt and Cook, 1990; Stambler *et al.*, 1991; McAuley, 1994; Muller-Parker *et al.*, 1994; McAuley and Cook, 1994), and reduces ammonium uptake rates (Yellowlees *et al.*, 1994). Ammonium assimilation is assumed to involve the coupled glutamine synthetase/2-oxoglutarate amido transferase (GS/GOGAT) pathway. At present, GS but not GOGAT has been detected in zooxathellae (Summons and Osmond, 1981; Wilkerson and Muscatine, 1984), although azaserine, a potent inhibitor of GOGAT, prevents ammonium uptake in intact corals (Rahav *et al.*, 1989), and the presence of GS/GOGAT activity is supported by the data of Summons *et al.* (1986).

In contrast to marine symbioses, in the symbiosis between the freshwater cnidarian *Hydra viridissima* (green hydra) and *Chlorella* algae, the host rather than the algal symbionts may be responsible for assimilation of ammonium. Ammonium assimilation by maltose-releasing *Chlorella* is inhibited at the low pH that stimulates maltose release (Rees, 1989). Recent evidence suggests that in symbiosis the algae are carbon- rather than nitrogen-limited, in that *in vivo* release of maltose consumes considerable amounts of photosynthetically fixed carbon that would otherwise be used to assimilate ammonium into amino acids (McAuley, 1992). Treatment of the intact symbiosis with the photosynthetic inhibitor DCMU does not cause ammonium excretion into the medium, and rates of ammonium uptake by freshly isolated symbionts are only 40% of those of the intact symbiosis (Rees, 1986; McAuley, 1990). Finally, the hosts possess GS, and levels of GS activity are higher in symbiotic than aposymbiotic animals (Rees, 1986).

Although green hydra do not normally excrete ammonium into the medium, release can be induced by treatment with methionine sulphoximine (MSX), which inhibits GS in intact hydra (Rees, 1986). However, the effect of MSX on algal GS has not been tested, because of the difficulty in isolating sufficient numbers of symbionts uncontaminated by host material. This paper describes the effects of inhibitors of enzymes of ammonium assimilation on perturbation of animal and algal internal pools of glutamate and glutamine, amino acids closely associated with ammonium assimilation (Miflin and Lea, 1976). Recovery of symbiotic algae after SDS washing, which is necessary to remove host contamination, is low (McAuley, 1986a), but unlike assays for enzyme activity, measurement of free amino acid pools by HPLC requires only small numbers of algae (the equivalent of 10^4 cells or fewer per sample injection). Uptake of MSX by algae *in vitro* and in the intact symbiosis was also measured using HPLC.

Materials and Methods

Maintenance of organisms

Green hydra of the European strain (EE hydra) were grown in unbuffered 'M' solution (Muscatine and Lenhoff, 1965) at 15°C in constant light (60 μmol photons m^{-2} s^{-1} PAR). Cultures were fed each Monday, Wednesday, and Friday with freshly hatched brine shrimp. All hydra used in experiments had not been fed for the previous 3 days to allow complete digestion of food. Cultures of the 3N813A strain of maltose-releasing *Chlorella*, grown in Kessler's medium (Kessler *et al.*, 1963), pH 6.3, were maintained in a shaking, illuminated incubator in growth conditions similar to those of hydra cultures.

Isolation of symbiotic algae from hydra

The SDS-washing technique (McAuley, 1986a) was used to isolate symbiotic *Chlorella* algae from hydra.

Determination of numbers of algae per digestive cell

Five gastric regions of hydra were isolated in a drop of macerating fluid (David, 1973). After 10 min, pieces of hydra were teased apart into individual cells and examined using phase contrast microscopy. Numbers of algae were determined in 100 randomly selected digestive cells in each preparation.

Measurement of ammonium uptake

Ammonium uptake was measured as depletion from the medium of either hydra or algae. Duplicate samples (200 μl) were taken at the beginning and end of experiments, and amounts of ammonium were determined with a scaled-down hypochlorite-nitroprusside colometric assay (Rees, 1986). After reagents were added, samples were incubated for 20 min in darkness, then absorbence was read at 630 nm and compared to that of ammonium sulphate standards (reagents and standards from Sigma Chemical Company).

Inhibitors

Methionine sulphoximine (MSX), an inhibitor of GS (Ranzio *et al.*, 1969; Meister, 1974), and azaserine (AZS), an inhibitor of 2-oxoglutarate amido transferase (GOGAT) (Wallsgrove *et al.*, 1977; Elrifi and Turpin, 1986), were purchased from the Sigma Chemical Company.

Stock solutions were stored at 4°C and routinely tested by measuring their effectiveness in inhibiting ammonium uptake by cultured 3N813A cells. In all experiments, MSX was used at a final concentration of 200 μM and AZS at 1 mM.

Extraction and measurement of free amino acid pools

Samples of algae, or of animal homogenates centrifuged at 1000 × g for 5 min to remove algae, were added to absolute ethanol to give 80% ethanol (v:v) and extracted for 24 h at 4°C. Protein contents of homogenates were determined using the Bradford method (Bradford, 1976). In some cases, algal samples were filtered at low vacuum through Whatman GF/C filters, which were then extracted twice in 80% ethanol. Extracts were dried *in vacuo* and resuspended in an appropriate volume of 12.5 μM α-amino-butyric acid (AABA), which acted as an internal standard. Amino acid contents of aliquots of extracts were determined by *o*-phthaldialdehyde pretreatment and reverse-phase HPLC as previously described (McAuley, 1992). MSX but not AZS was detectable using this system; the MSX peak occurred 0.8 min after that of serine.

Glutamine and methylamine uptake

Glutamine uptake was determined from cultured 3N813A cells resuspended in 10 mM MES buffer pH 7 at a density of 5×10^7 cells ml^{-1}. After 30 min of preincubation, the assay was started by adding 2–20 μM glutamine containing 3.7 μBq [U-^{14}C]-glutamine (Amersham International; specific activity 9.25 GBq mmol^{-1}) with or without additional 20 μM AZS. At intervals of 1, 2, and 3 min after addition of radioactivity, 200-μl samples were filtered onto Whatman GF/C filters at low vacuum and washed with 20 ml distilled water. Filters were dried in scintillation vials, 10 ml of scintillation fluid was added (McAuley, 1988), and samples were counted on an LKB 1214 Rackbeta scintillation counter. V_{max} and K_M were determined from Lineweaver Burke double reciprocal plots of uptake rates against substrate concentration.

Uptake of [^{14}C]-methylamine was determined by the same method as glutamine uptake, except that [^{14}C]-methylamine (Amersham International; specific activity 2.22 GBq mmol^{-1}) was added to give a final concentration of 5 μM. At intervals, 100-μl samples were filtered onto Whatman GF/C filters and radioactivity was determined as described above.

Enzyme assay

About 100 hydra, previously starved for 3 days, were homogenized in extraction buffer as described by Rees (1986). The homogenate was centrifuged at 13000 × g for 5 min at 4°C, and the supernatant was assayed for GOGAT activity by determining the rate of oxidation of NAD(P)H at 340 nm by the method of Bhandari and Nicholas (1981). Blanks were run without addition of α-ketoglutarate.

Results

Effect of ammonium on symbionts in intact hydra

Incubation of EE hydra for 7 days in M solution supplemented with 50 μM ammonium chloride had no effect on the size of the glutamate and glutamine pools of their algal symbionts (Table I). However, significantly more algae (one-way ANOVA, $P < 0.05$, $n = 6$ independent experiments) were present in digestive cells of hydra maintained in ammonium-supplemented medium (24.94 ± 1.71 SD) than in controls (22.58 ± 1.48).

Effect of MSX and AZS on ammonium uptake

As previously observed (Rees, 1986; McAuley, 1990), MSX not only inhibited ammonium assimilation but also caused release of ammonium from both intact hydra and cultured 3N813A algae (Table II). AZS inhibited ammonium assimilation but did not cause release in 3N813A algae; ammonium release by green hydra treated with AZS was seven times lower than that observed with MSX treatment.

Effect of MSX and AZS on internal pools of glutamate and glutamine in 3N813A

3N813A algae were incubated with either MSX or AZS in the absence or presence of a nitrogen source (either glutamine or ammonium), and amino acid pools were compared to those of untreated controls (Table III). MSX and AZS had distinct effects on internal glutamine and glutamate pools irrespective of the nitrogen supply, and

Table I

Effect of incubation of intact hydra in ammonium on glutamate and glutamine pools of algal symbionts

	Controls	Ammonium treated
Glu	574.1 ± 38.0	578.9 ± 56.7
Gln	150.3 ± 18.5	146.7 ± 29.3
Gln:Glu	0.26 ± 0.03	0.26 ± 0.06

EE Hydra were starved for 7 days in M solution ±50 μM ammonium chloride; incubation solutions were changed every day. After 7 days, hydra were washed five times in large volumes of M solution and homogenized, and amino acid pools in SDS-washed freshly isolated symbionts were determined as described in Materials and Methods. Values are the means ± SD of amino acid content (amol cell^{-1}) of glutamate and glutamine pools determined from three independent experiments.

Table II

Effect of MSX and AZS on uptake (+) or release (−) of ammonium by intact green hydra and 3N813A algae

	Hydra (nmol hydra^{-1} h^{-1})	3N813A (fmol cell^{-1} h^{-1})
Control	+0.192 ± 0.045	+1.51 ± 0.35
MSX	−0.220 ± 0.036	−0.86 ± 0.25
AZS	−0.031 ± 0.006	+0.05 ± 0.03

Hydra were preincubated in MSX or AZS for 24 h and washed twice in large volumes of growth medium before being placed in new medium; 3N813A algae (5×10^7 cells ml^{-1}) were preincubated for 30 min in 10 n*M* MES pH 7. Uptake of ammonium was measured by the difference in ammonium content of aliquots taken immediately after addition of ammonium chloride to give a concentration of 50 μM (hydra) or 100 μM (algae) and of aliquots taken after 1 h. Figures are the means ± SD of three (hydra) or four (algae) independent experiments. All treatment values are significantly different from controls (one-way ANOVA, $P < 0.001$).

these effects were consistent with inhibition of GS or GOGAT respectively. MSX significantly decreased glutamine and AZS significantly decreased glutamate in all treatments. In algae without a nitrogen source or supplied with glutamine, MSX also significantly decreased glutamate pools; in algae without a nitrogen source or supplied with ammonium, AZS significantly increased glutamine pools.

Supply of nitrogen also affected glutamine and glutamate pools. Glutamine increased in algae supplied with ammonium and glutamine, and glutamate increased in algae supplied with glutamine (one-way ANOVA, $P < 0.05$). Tukey post-hoc tests showed that the size of the glutamine pools was similar in all MSX and all AZS treatments ($P > 0.05$). The size of the glutamate pools was also similar in all AZS treatments ($P > 0.05$), but glutamate levels were higher in algae treated with MSX in the presence of glutamine than in the presence of ammonium or the absence of a nitrogen source.

Effect of MSX and AZS on internal pools of glutamate and glutamine in freshly isolated symbionts and in the intact symbiosis

The effect of maltose release on the changes the two inhibitors produce in glutamate and glutamine pools was tested on freshly isolated symbionts treated at pH 5 and at pH 7. Maltose release is pH dependent (Cernichiari *et al.*, 1969) and occurs at the former but not the latter pH.

When freshly isolated symbionts were treated with MSX and AZS at pH 7, the effects were similar to those seen when 3N813A algae were treated without a nitrogen source: MSX caused a significant decrease in glutamine and glutamate; AZS caused a significant decrease in glutamate and an increase in glutamine (Table IV). At pH 5, AZS treatment also caused a significant decrease in glutamate and an increase in glutamine, but whereas MSX treatment decreased glutamate, glutamine levels tended to increase, although this trend was not significant. Uptake of MSX at pH 5 was considerable (over 3000 amol cell^{-1}), and the large MSX peak, adjacent to that of glutamine, may have interfered with detection of glutamine. When an amount of MSX equivalent to that found in the algae was added to a sample containing 10 pmol glutamine, the apparent amount of glutamine detected by HPLC increased 30%. The smaller MSX peak at pH 7 did not interfere with glutamine detection.

Table III

Effect of MSX and AZS on free glutamate and glutamine pools of 3N813A algae

	Control	MSX	AZS
−N			
Glu	379.9 ± 24.2	295.9 ± 40.3*	68.4 ± 20.2*
Gln	77.3 ± 10.3	11.4 ± 2.3*	437.7 ± 72.8*
+NH4			
Glu	335.0 ± 14.2	300.4 ± 22.1	50.5 ± 13.1*
Gln	166.1 ± 47.2	11.5 ± 5.5*	438.4 ± 51.0*
+Gln			
Glu	625.4 ± 99.0	407.1 ± 62.7*	56.8 ± 6.3*
Gln	330.1 ± 62.3	49.7 ± 16.4*	495.9 ± 91.6

Algae were incubated with MSX or AZS or in 10 m*M* MES buffer pH 7 for 30 min before addition of 100 μM ammonium chloride, glutamine, or an equivalent volume of distilled water (−N). After 1 h, aliquots were filtered at low vacuum onto Whatman GF/C filters, washed, and extracted for amino acid analysis. Figures are the means ± SD of amino acid content (amol cell^{-1}) of glutamate and glutamine pools determined from three independent experiments.

* Significantly different from control, one-way ANOVA, $P < 0.05$.

Table IV

Effect of MSX or AZS on free glutamate and glutamine pools of freshly isolated symbiotic algae

	Control	MSX	AZS
pH 7.0			
Glu	334.1 ± 62.3	182.2 ± 29.1*	63.6 ± 13.1*
Gln	120.1 ± 27.2	50.9 ± 12.7*	297.1 ± 102.3*
pH 5.0			
Glu	156.0 ± 4.5	84.0 ± 22.1*	62.1 ± 14.7*
Gln	60.6 ± 6.5	95.4 ± 23.1	89.2 ± 11.4*

Algae were incubated with MSX or AZS or in 10 m*M* MES buffer only (controls) for 90 min, and then aliquots were filtered at low vacuum onto Whatman GF/C filters, washed, and extracted for amino acid analysis. Figures are the means ± SD of amino acid content (amol cell^{-1}) of glutamate and glutamine pools determined from three independent experiments.

* Significantly different from control value, one-way ANOVA, $P < 0.05$.

Treatment of freshly isolated symbionts at pH 5 caused a significant reduction in both glutamate and glutamine pools compared to those at pH 7 (one-way ANOVA, $P < 0.05$).

Incubation of intact green hydra in MSX caused no significant change in the glutamate or glutamine pools of either the symbiotic algae or the host (Table V). Only 16.2 ± 30.2 SD amol cell^{-1} MSX was detected in algal pools after 24-h treatment of intact hydra, compared to high levels in freshly isolated symbionts after only 90 min of treatment (3126.0 ± 227.6 amol cell^{-1} in freshly isolated symbionts treated at pH 5; 495.3 ± 175.7 amol cell^{-1} in freshly isolated symbionts treated at pH 7). Treatment of intact hydra with AZS increased glutamine pools in both hydra and algae, although the increase was significant only in hydra. AZS treatment did not cause a reduction in either host or algal glutamate pools, in contrast to the consistent effect of AZS on glutamate pools measured in both freshly isolated symbionts and 3N813A irrespective of pH or nitrogen supply.

Effect of MSX and AZS on glutamine and methylamine uptake by 3N813A algae

[^{14}C]-glutamine uptake by 3N813A algae was competitively inhibited by a low concentration of AZS (Fig. 1). K_M was 17.3 μM in the absence of AZS and 28.6 μM in the presence of AZS. In both cases, V_{max} was 250 amol glutamine cell^{-1} h^{-1}.

There was linear uptake of the ammonium analog [^{14}C]-methylamine in the presence of both MSX and AZS, although uptake rates were reduced by 25.1% and 33.4% respectively, in comparison to controls (Fig. 2). In contrast, ammonium chloride inhibited methylamine uptake by 98% for the first 4 min, although the uptake increased gradually thereafter. Calculations based on ammonium uptake rates in Table I indicated that the algae would have greatly depleted ammonium concentration at the time that uptake of methylamine began to increase.

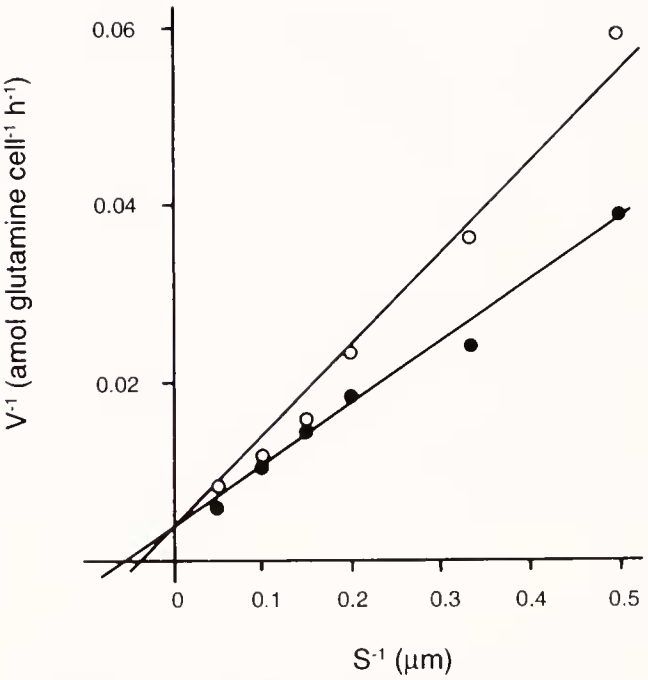

Figure 1. Effect of the absence (● —— ●) and presence (○ —— ○) of AZS on the apparent K_M value of the glutamine transport system of 3N813A algae. Algae were preincubated in 10 mM MES buffer pH 7 for 30 min before addition of 2–20 μM [^{14}C]-glutamine with or without 20 μM AZS. Uptake of glutamine and K_M and V_{max} was determined as described in Materials and Methods.

Table V

Effect of incubating hydra in MSX or AZS on free glutamate and glutamine pools of hydra (pmol μg protein^{-1}) and symbiotic algae (amol cell^{-1})

	Control	MSX	AZS
Hydra			
Glu	24.3 ± 10.4	24.3 ± 12.8	25.1 ± 12.0
Gln	1.3 ± 0.4	1.8 ± 0.9	5.9 ± 3.0*
Algae			
Glu	551.3 ± 82.7	571.5 ± 143.6	554.8 ± 94.6
Gln	146.2 ± 54.3	156.6 ± 65.8	224.1 ± 128.1

One hundred and twenty hydra were incubated in MSX or AZS for 24 h, washed five times in large volumes of M solution and homogenized, and amino acid pools in homogenized hydra and in SDS-washed freshly isolated symbionts were determined as described in Materials and Methods. Figures are the means ± SD of six independent experiments.

* Significantly different from control value, one-way ANOVA, $P < 0.05$.

Uptake of glutamine by freshly isolated symbionts

Short-term experiments showed that incubation of freshly isolated symbionts in 100 μM glutamine caused an increase in internal glutamine and glutamate pools at both pH 5 and pH 7 (Fig. 3).

GOGAT activity

Neither NADH- or NADPH-specific GOGAT activity was detectable in hydra tissue.

Discussion

Several lines of evidence suggest that assimilation of ammonium in the green hydra–*Chlorella* symbiosis is due to GS in the animal host rather than in the algal symbiont. Incubation of green hydra in medium supplemented with ammonium chloride caused a small, significant increase in numbers of algae per host digestive cell, but there was

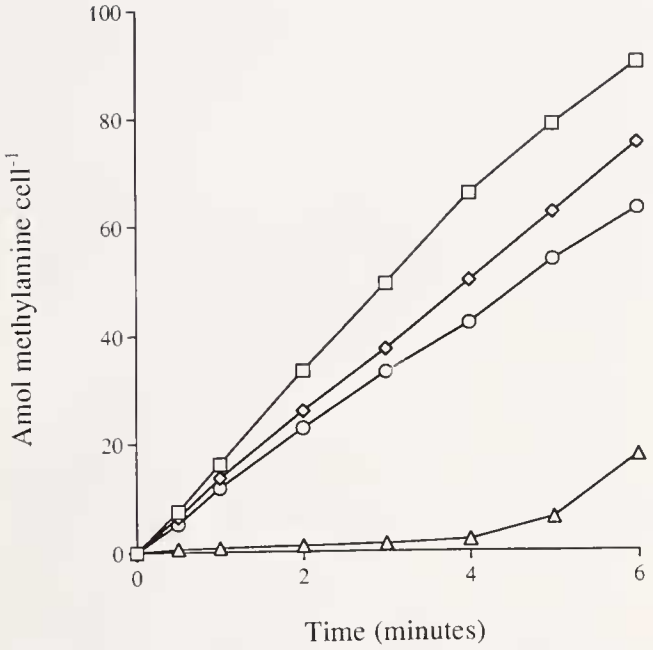

Figure 2. Effect of MSX, AZS, and ammonium on uptake of [^{14}C]-methylamine by 3N813A algae. Algae were preincubated in 10 m*M* MES buffer pH 7 for 30 min before addition of 5 μM [^{14}C]-methylamine together with either 10 μM MSX (——), 10 μM AZS (○ —— ○), 10 μM ammonium chloride (△ —— △), or an equivalent volume of buffer only as control (□ —— □) to give a final density of 5×10^7 cells ml^{-1}. At intervals, 100-μl samples were filtered and radioactivity was determined.

no effect on glutamine and glutamate pools or on the Gln:Glu ratio of the algae (Table I), the latter being an indicator of nitrogen sufficiency in microalgae (Flynn *et al.*, 1989). In marine symbioses, in which it is believed that symbionts are able to assimilate ammonium from seawater, ammonium supplementation not only causes large increases in the population density of symbionts (Muscatine *et al.*, 1989; Hoegh-Guldberg and Smith, 1989; Dubinsky *et al.*, 1990; Fitt and Cook, 1990; Stambler *et al.*, 1991; Muller-Parker *et al.*, 1994), but, in a coral and a hydroid, also increases the size of internal glutamine pools of symbionts and hence their Gln:Glu ratio (McAuley, 1994; McAuley and Cook, 1994).

Further, although MSX inhibits ammonium uptake by intact hydra (Rees, 1986; McAuley, 1990; Table II, this paper), almost no MSX was detected in symbiotic algae isolated from hydra that had been incubated in MSX for 24 h, and there was no change in algal internal pools of glutamate and glutamine. In contrast, freshly isolated symbionts treated with MSX rapidly accumulated large amounts of the inhibitor, and MSX treatment caused predictable changes in glutamate and glutamine pools of freshly isolated symbionts and cultured 3N813A algae in a variety of conditions (Tables III and IV).

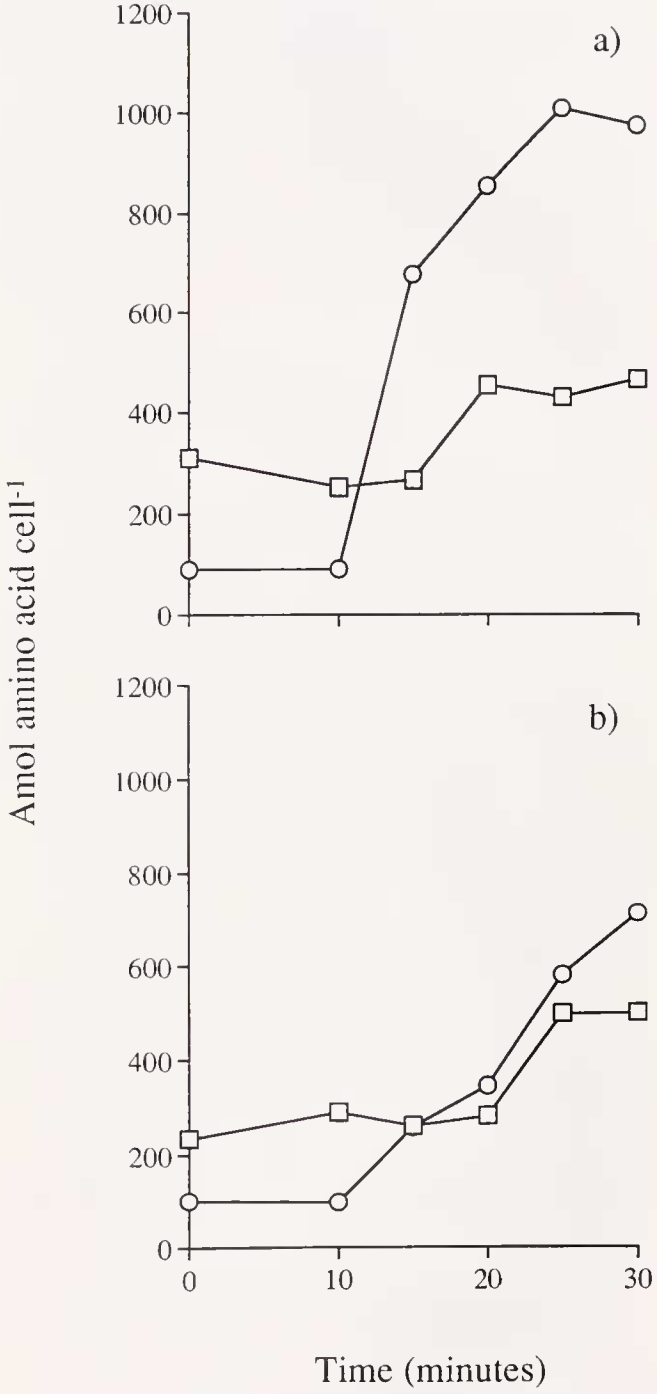

Figure 3. Effect of glutamine uptake on internal pools of glutamine (○ —— ○) and glutamate (□ —— □) in freshly isolated symbionts. Freshly isolated symbionts (10^7 cells ml^{-1}) were preincubated for 30 min in 10 m*M* McIlvaine's buffer at either pH 5 (a) or pH 7 (b). Ten minutes after the start of the experiment, glutamine was added to give a concentration of 100 μM. Samples (200 μM) taken at each time point were filtered onto GF/C disks under low vacuum, washed with 20 ml ice-cold distilled water, and extracted for amino acid analysis.

In addition to inhibiting GS, MSX has been shown to be a noncompetitive inhibitor of active ammonium transport in *Anabaena flos-aquae* (Turpin *et al.*, 1984). In 3N813A, both MSX and AZS caused a degree of inhibition of uptake of methylamine (Fig. 2), a structural analog of ammonium that is taken up by the same transport system (Hackette *et al.*, 1970; Pelley and Bannister, 1979; Smith, 1982; Wright and Syrett, 1983). However, if both MSX and AZS caused noncompetitive inhibition of entry of ammonium into symbionts, release of ammonium from the intact symbiosis would presumably be the same in both treatments. Instead, release was seven times higher in hydra treated with MSX than in hydra treated with AZS (Table II). As shown in 3N813A cells, MSX blocks assimilation of ammonium into glutamine *via* GS (Ranzio *et al.*, 1969; Meister, 1974), leading to low glutamine pools and release of ammonium. AZS blocks subsequent metabolism of glutamine to glutamate *via* GOGAT (Wallsgrove *et al.*, 1977; Elrifi and Turpin, 1986), leading to accumulation of glutamine, low glutamate pools, and no ammonium release. That hydra release more ammonium when treated with MSX than with AZS is consistent with inhibition of GS activity pathways rather than with noncompetitive inhibition of ammonium transport into the algae. Because very little MSX was detected in symbionts after treatment of intact hydra, the ammonium release observed during MSX treatment of the intact symbiosis must have been due to inhibition of host GS.

No GOGAT was detected in crude, alga-free extracts of homogenized green hydra. However, treatment of intact hydra with AZS, an inhibitor of GOGAT, prevented uptake of ammonium from the medium by the intact symbiosis and caused a significant increase in host glutamine pools (Tables II and V). This may be explained if glutamine resulting from activity of host GS was taken up by the algae and converted into glutamate *via* GOGAT. Uptake of glutamine by algae would also explain why MSX treatment did not cause an increase in host glutamine pools.

AZS treatment may have affected this coupling in two ways. First, AZS may have specifically inhibited algal GOGAT, although AZS treatment of the intact symbiosis did not cause the reduction in glutamate and increase in glutamine consistently observed in 3N813A or in freshly isolated symbionts. Second, AZS, which competitively inhibited uptake of glutamine in 3N813A algae (Fig. 1), prevented entry of glutamine into either the perialgal vacuole or the symbiotic algae themselves. In either case, the glutamine transport system must be specific for glutamine and AZS but not MSX. Given that symbionts accumulated MSX *in vitro* but not *in hospice*, it is possible that transport systems in the host perialgal vacuolar membrane differentially control entry of substances into the perialgal vacuole.

Supply of nitrogen to algal symbionts as glutamine or other amino acids rather than as ammonium may be important because a supply of carbon is required for assimilation of ammonium into amino acids (Turpin, 1991), and symbionts release a high proportion of fixed carbon to their host in the form of maltose (Mews, 1980). In cultured 3N813A, maltose synthesis and release inhibits ammonium uptake because it diverts photosynthetically fixed carbon from assimilation of ammonium into amino acids (McAuley, 1992). Maltose release is stimulated by low pH, and ammonium uptake by freshly isolated symbionts and cultured maltose-releasing algae falls as pH is reduced and maltose release increases (Rees, 1989). Below a critical pH value, the algae actually begin to release ammonium, not only because fixed carbon is diverted from the TCA cycle to maltose release, but also because amino acids may be deaminated to provide carbon skeletons for maltose synthesis (Rees, 1990).

Given that high rates of maltose release and ammonium assimilation are incompatible, amino acids may provide an alternative source of nitrogen for algae in symbiosis with green hydra. Freshly isolated symbionts have active amino acid transport systems (McAuley, 1986b), and algae in the intact symbiosis can assimilate a variety of amino acids supplied by host feeding (McAuley, 1987b, 1988, 1991). Further, the rate of glutamine uptake by freshly isolated symbionts peaks between pH 4 and 5, the same range over which release of maltose is maximum (McAuley, 1991). Maltose-releasing symbionts using glutamine as a nitrogen source would require carbon skeletons in the form of 2-oxoglutarate to produce two molecules of glutamate from each molecule of glutamine. But even if one molecule of glutamate was deaminated, the carbon recycled to 2-oxoglutarate, and the ammonium released to be reassimilated by the host, the algae would still gain a new molecule of glutamate for each molecule of glutamine taken up. Short-term experiments showed that uptake of glutamine by freshly isolated symbionts at both pH 5 (with maltose release) and pH 7 (without maltose release) caused an increase in the size of internal glutamate pools (Fig. 3). Although the increase in glutamate pools was similar, it should be noted that the rate of glutamine uptake by freshly isolated symbionts is more than 5 times higher at pH 5 than at pH 7 (McAuley, 1991).

This scheme is qualitatively but not quantitatively different from that in marine algal–invertebrate symbioses, in which symbiotic zooxanthellae appear to assimilate ammonium *via* coupled GS/GOGAT (for review, see Spencer-Davies, 1992). In green hydra, although both GS and GOGAT are involved in ammonium assimilation, they may be located in different compartments, and the necessity for glutamine to traverse the perialgal vacuole

would produce a point at which nitrogen supply to the algae could be regulated.

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